Optimization of the Sliced Testis Steroidogenesis Assay

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RTI International

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Background

- The DRP named several possible procedures to study steroidogenesis and its' ability to characterize the endocrine effects of various environmental contaminants, industrial substances and pesticides
  - Tissue culture
    - Purified cell preparations
    - Cell lines
  - Ovarian *in vitro* steroidogenesis assay
  - Testicular *in vitro* steroidogenesis assay

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Why Sliced Testis Assay?

- Minimal Cost
- Quick
- Uses Standard Laboratory Equipment
- Basic Laboratory Training
- Stable Preparation
- Uses Reduced Number of Animals
- Can be Standardized
- Well-defined endpoint in testosterone concentration
- Can be modified to use intermediate hormonal endpoints

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Optimization of the Sliced Testis Assay

This was planned to be Implemented in Two Phases

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Prototypical Sliced Testis Assay

- Testes are weighed and placed in DPBS buffer
- Each testis is sliced along the longitudinal axis into slices of proper weight
- Slices are placed with 5mL of media
- Incubated at 34ºC in 5%CO_2 and 95% air on a shaker

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Sliced Testis Assay

- At first time-point –baseline – media is removed and discarded
- Fresh media is added and an aliquot is collected
- Half of the samples are challenged with a stimulant, such as hCG
- Aliquots are collected at 1, 2, 3 and 4 hours post-challenge
- Samples are analyzed for testosterone concentration in a RIA assay

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Technical Flow Illustration of Sliced Testis Assay

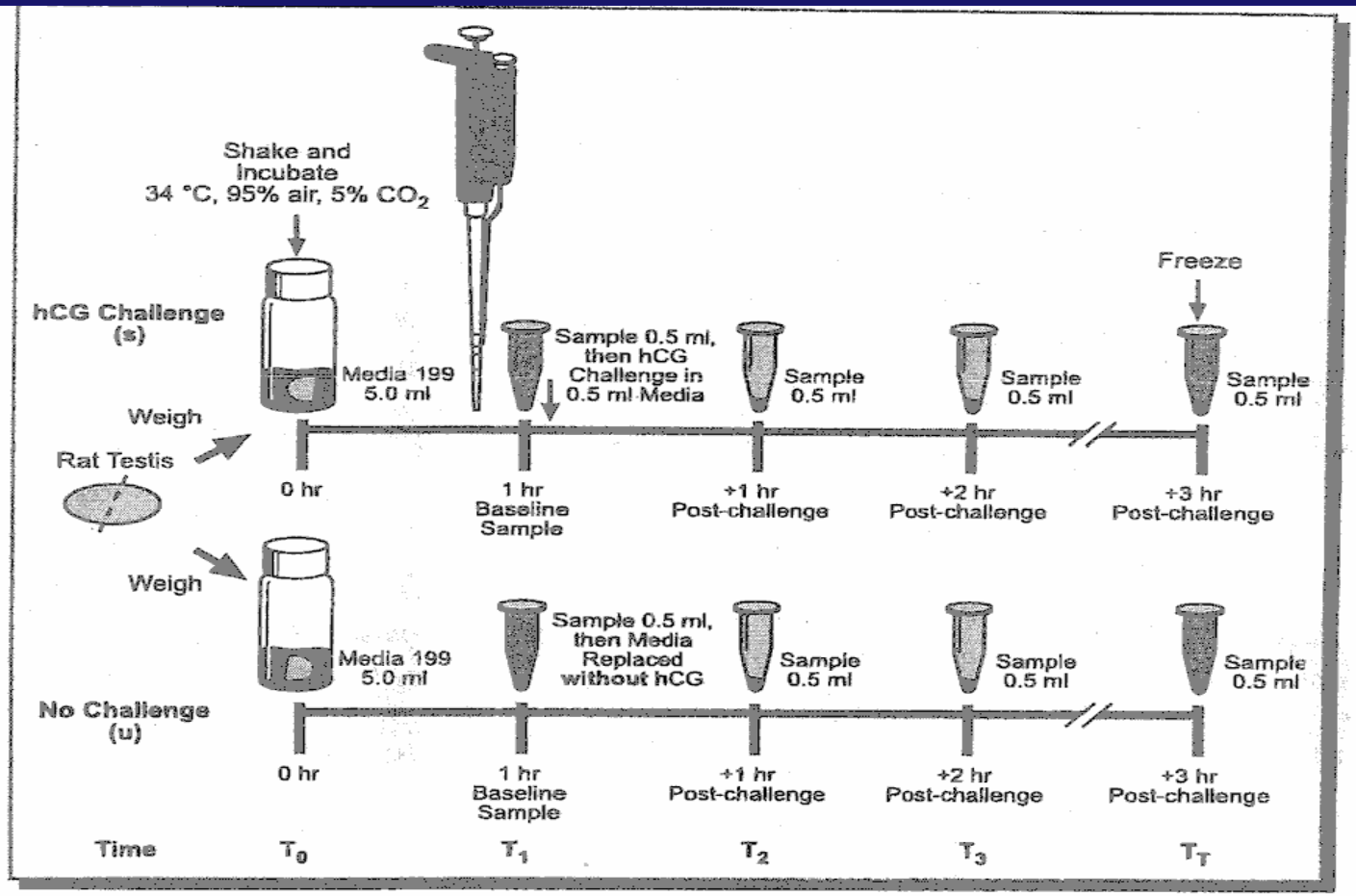

Figure 2. Technical Flow Illustration of the Sliced Testis Steroidogenesis Assay

Phase 1: Preliminary Experimental Phase

- Establish whether a given level of each factor affects assay performance
- The factors are unlikely to have an interaction or at best a minimal interaction with another experimental factor
- An effect of one of these factors would require additional verification experiments after sensitivity analysis

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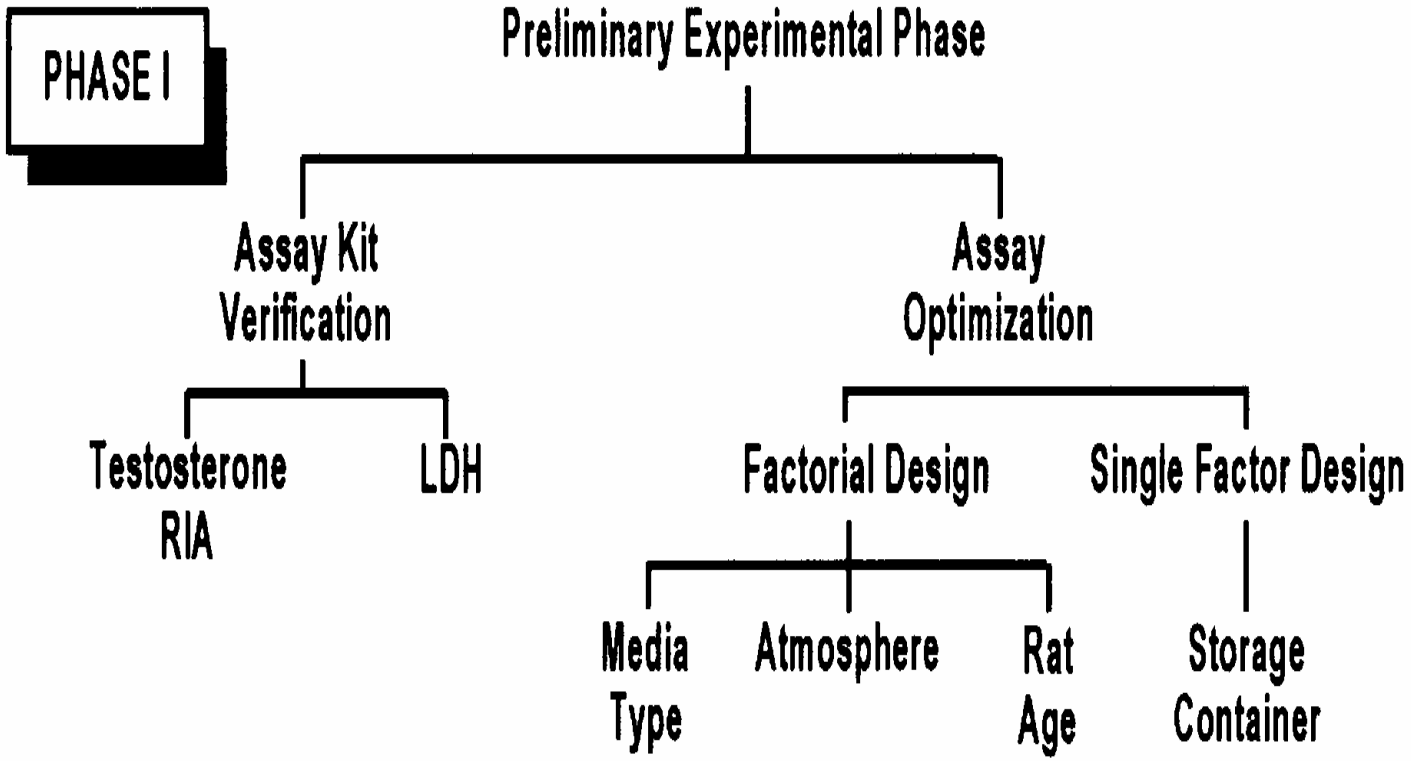
Phase I Design



Phase II: Primary Experimental Phase

- Factors that may affect assay performance were tested
- These factors were divided into four sections where each section was composed of factors that might produce interactions

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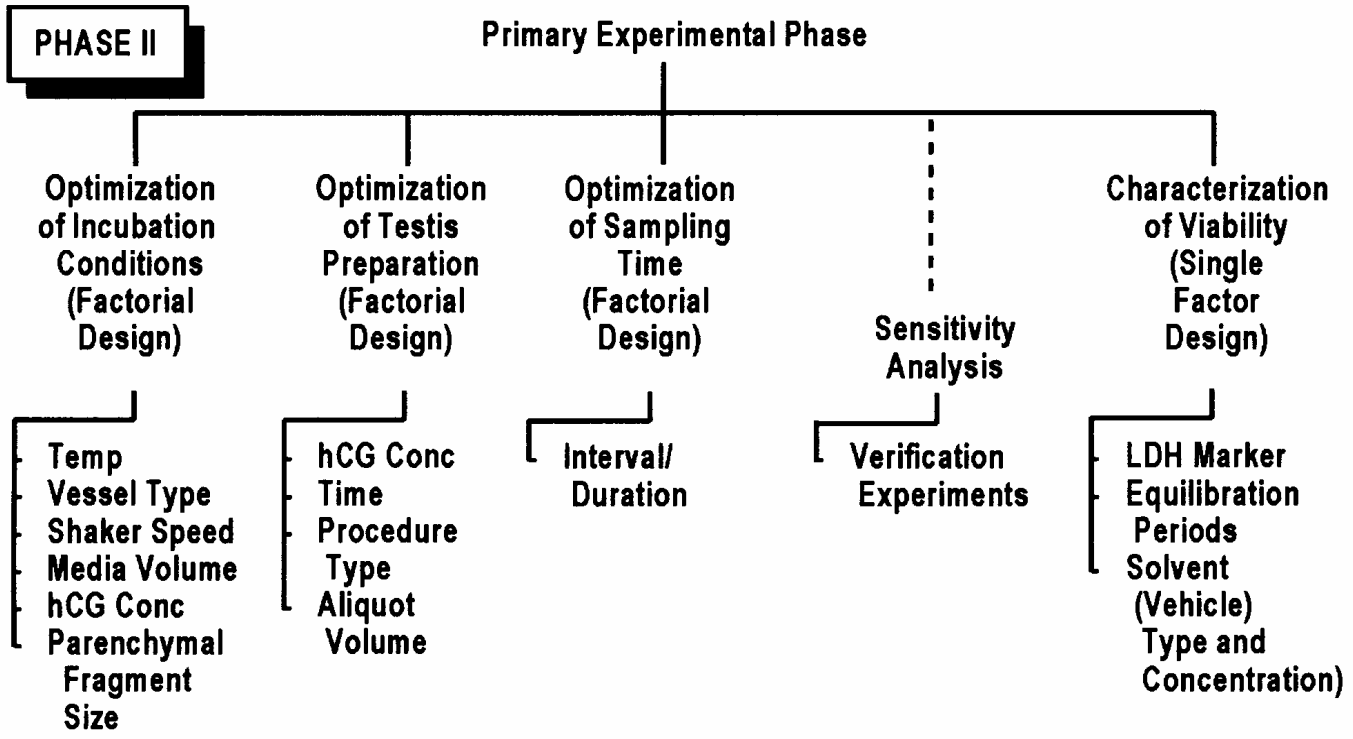
Phase II Design



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Testosterone Radioimmunoassay

- RIA
- Used to measure testosterone concentration of the aliquots taken at various time-points
- Commercial kit
- Utilizes 125 I-testosterone and a testosterone-specific antibody affixed to tubes

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Testosterone RIA

- Verification of the assay using Media 199 without phenol red
- Determination of assay accuracy, sensitivity, precision and parallelism

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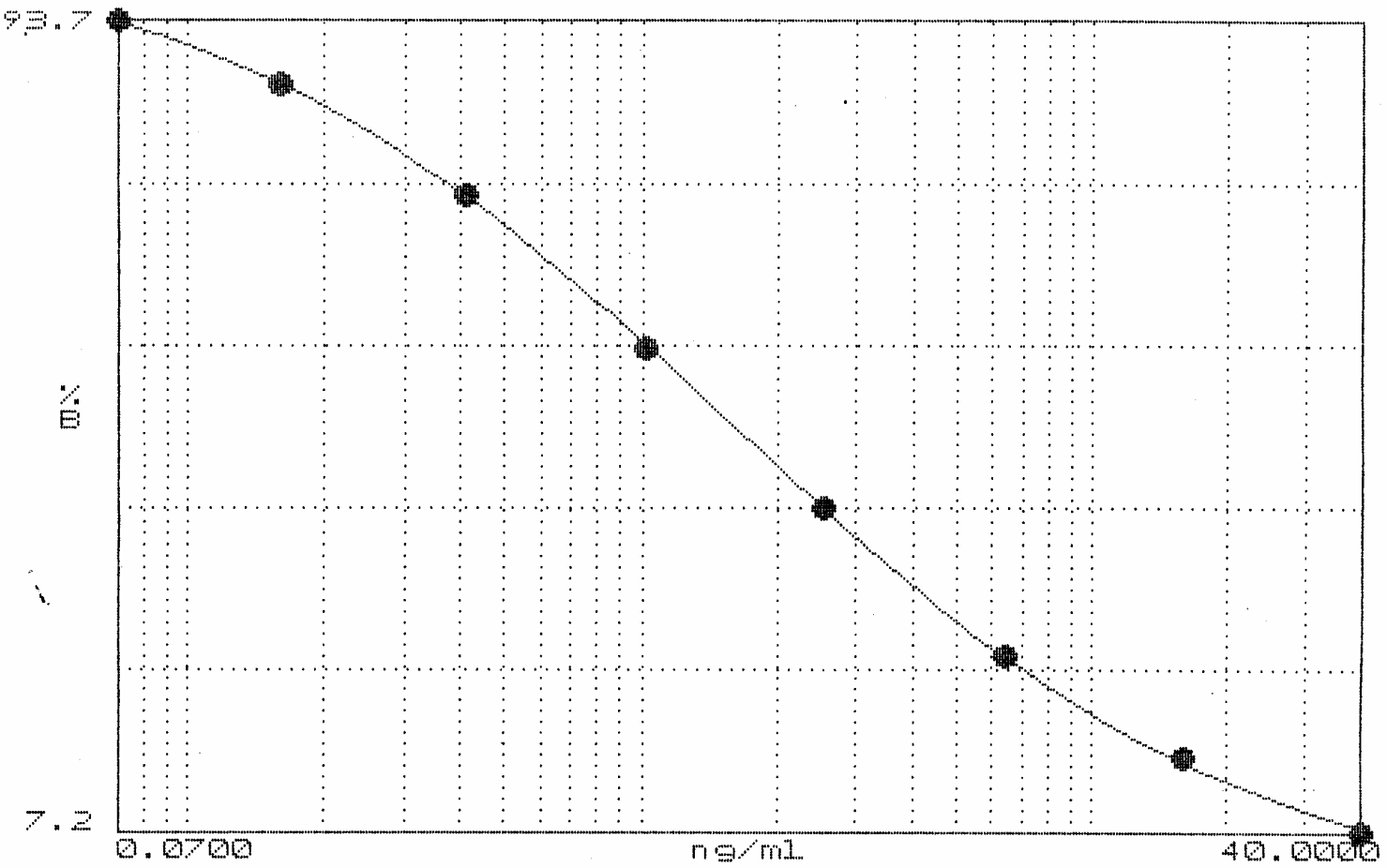
Assay Type: RIA %REF. BOUND



STD #	CPM	DEFINED DOSE	%B/F	CALC.DOSE	% DIFF
1	13974	0.07000	93.66	0.06874	-1.80
2	12999	0.16000	86.86	0.15975	-0.16
3	11321	0.41000	75.15	0.40520	-1.17
4	8976	1.02000	58.78	1.04723	2.67
5	6516	2.56000	41.61	2.56859	0.34
6	4300	6.40000	26.14	6.37291	-0.42
7	2732	16.00000	15.20	15.23172	-4.80
8	1592	40.00000	7.24	42.18885	5.47

Testosterone RIA Validation with M-199

Tstosterone RIA Percent Recovery			
	50 µl	25 µl	10 µl
+ 8 ng/ml	113.8	133.3	149.0
+ 2 ng/ml	129.5		
0.5 ng/ml	146.5		

Testosterone RIA Assay Parallelism			
	50 µl	25 µl	10 µl
+ 8 ng/ml	9.10 ng/ml	10.66 ng/ml	11.92 ng/ml

The Index between 50 and 25 10 µl was 117.1%, between 25 and 10 µl was 111.8% and between 50 and 10 µl was 130.99%.

Testosterone RIA Intra-assay CV				
	Number	50 µl	25 µl	10 µl
Unspiked M199	2	Blanks		
+ 8 ng/ml	10	5.24%	8.64%	7.68%
+ 2 ng/ml	10	6.09%		
+ 0.5 ng/ml	10	13.34%		

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Lactate Dehydrogenase (LDH) Assay

- Validation of the assay using Media-199 without phenol red
- Measuring the assay accuracy, sensitivity, and precision
- Performed at Laboratory Corporation of America

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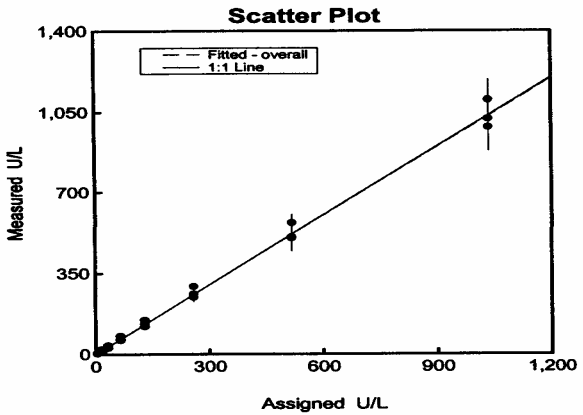
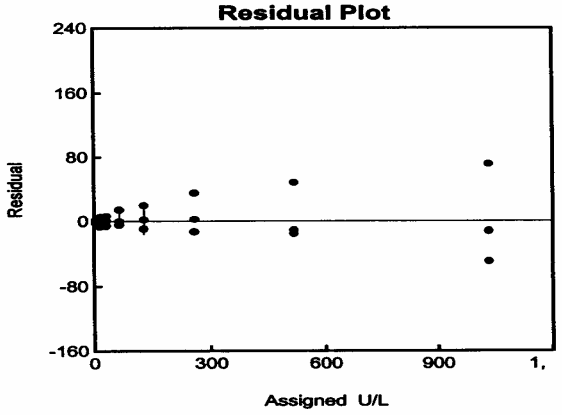
EP Evaluator

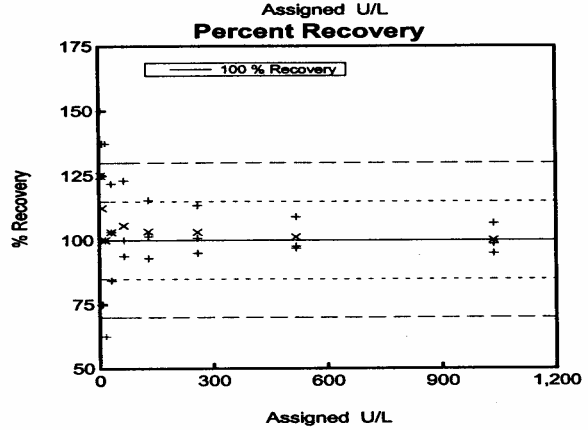
Preclinical -- LabCorp

LDH-Media

Instrument: Hitachi







Phase I

- Tested:
  - Media Type
    - Eagles MEM
    - RPMI-1640
    - Medium-199
  - Gaseous Atmosphere
    - 5% CO_2 / 95% air
    - 5% CO_2 / 95% O_2
    - Air
  - Rat Age
    - 11 weeks
    - 15 weeks
    - 22 weeks

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PHASE I: Experimental Design

Table 2. Summary of Experimental Factors for Phase 1 Optimization

Factor Identification	Units	Experimental Levels			Coded Experimental Levels		
		1	2	3	1	2	3
Media Type	NA	RPMI-1640	medium-199	Eagles-MEM	-1	0	+1
Gaseous Atmosphere	NA	5% CO_2 / 95% air	5% CO_2 / 95% O_2	air	-1	0	+1
Rat Age	wks	11	15	22	-1	0	+1

NA = not applicable.

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Based on Original-Scale Models Adjusted Mean Levels

Level of Independent Variable			Mean Levels of Dependent Variables: Without hCG Stimulation				Mean Levels of Dependent Variables: With hCG Stimulation			
Media (z1)	Gas (z2)	Age (z3)	Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4
RPM 1-1640 (-1)			3.55	5.05	6.14	6.99	4.52*	10.08*	18.11	25.30
Media 199 (0)			3.95	5.64	6.83	7.92	5.83	13.70	23.41	34.01
Eagles ME (+1)			3.67	5.24	6.62	7.75	5.77	13.34	21.38	31.26
5% CO_2 / 95% Air (-1)			3.72	5.16*	6.31**	7.50*	5.62	12.43*	20.40**	30.39**
5% CO_2 / 95% O_2 (0)			4.12	6.37	7.97	9.33	6.02	17.10	31.25	45.05
Air (+1)			3.34*	4.40**	5.30**	5.82**	4.49**	7.59**	11.25**	15.14**
11 Weeks (-1)			3.86**	5.53*	6.59*	7.67*	6.34	16.58	28.33	38.71
15 Weeks (0)			4.89	6.58	7.99	9.56	6.91	14.50	25.61	38.99
22 Weeks (+1)			2.43**	3.83**	5.01**	5.43**	2.88**	6.03**	8.97**	12.88**
-1	-1		3.07**	4.28**	5.44**	6.01**				
-1	0		4.06	6.00	7.11*	8.30				
-1	+1		3.51*	4.86**	5.88**	6.65**				
0	-1		4.07	5.24**	6.48**	7.60*				
0	0		4.85	7.63	9.43	11.03				
0	+1		2.94**	4.04**	4.57**	5.11**				
+1	-1		4.02	5.96	7.02*	8.89				
+1	0		3.44*	5.48*	7.38*	8.67				
+1	+1		3.55*	4.29**	5.46**	5.69**				
-1		-1					5.43**	15.00	28.51	38.78
-1		0					5.40**	9.68**	17.65**	25.55**
-1		+1					2.73**	5.55**	8.18**	11.58**
0		-1					7.68	20.02	34.14	46.02
0		0					7.05	14.90	26.88	42.62
0		+1					2.76**	6.17**	9.21**	13.40**
+1		-1					5.90*	14.71	22.34*	31.33*
+1		0					8.28	18.93	32.30	48.80
+1		+1					3.13**	6.37**	9.50**	13.67**

Shaded cell indicates highest mean estimated level.

* Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.05.

** Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.01.

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Phase I Optimizations

- Medium-199 without phenol red
- Gaseous atmosphere of 5% CO_2 / 95% O_2
- Rats that were 11 weeks of age showed results similar to those that were 15 weeks of age, therefore rats 11-15 weeks can be used in the assay

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Phase II

- To be tested:
  - Incubation Temperature
  - Incubation Vessel Type
  - Incubation Shaker Speed
  - Incubation Media Volume
  - hCG Concentrations
  - Testicular Fragment Size
  - Time Delay before starting the assay preparation
  - Organ Preparation Technique
  - Sample Aliquot Volume

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Table 6. Summary of Experimental Testis Preparation Factors for Optimization

Factor Identification	Units	Factor	Experimental Levels			Coded Experimental Levels		
			1	2	3	1	2	3
hCG Concentration	IU/ml	X5	0.01	0.1*	1	-1	0	+1
Time Delay	hr	X7	0.5	1*	2	-1	0	+1
Organ Preparation Technique	NA[a]	X8	Cold buffered saline	Warm buffered saline	Cold media*	-1	0	+1
Sample Aliquot Volume	ml	X9	0.125	0.25	0.5*	-1	0	+1

*Prototypical value.

[a] NA - not applicable.

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Table 4. Summary of Experimental Incubation Factors for Optimization

Factor Identification	Units	Factor Name	Experimental Levels					Coded Experimental Levels				
			1	2	3	4	5	1	2	3	4	5
Incubation Temperature	°C	X1	--	34*	--	37	--	--	-1	--	+1	--
Incubation Vessel Type	NA[a]	X2	scintillation vial*	test tube	--	--	--	-1	+1	--	--	--
Incubation Shaker Speed	NA	X3	--	none	low	high *	--	--	-1	0	+1	--
Incubation Media Volume	ml	X4	--	2.5	5*	10	--	--	-1	0	+1	--
hCG Concentration	IU/ml	X5	0.001	0.01	0.1*	1	10	-2	-1	0	+1	+2
Fragment Size	mg	X6	25	50	125	250*	--	-0.8	-0.6	0	+1	--

* Prototypical value.
[a] NA - not applicable.

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Technical Flow of Sliced Testis Assay

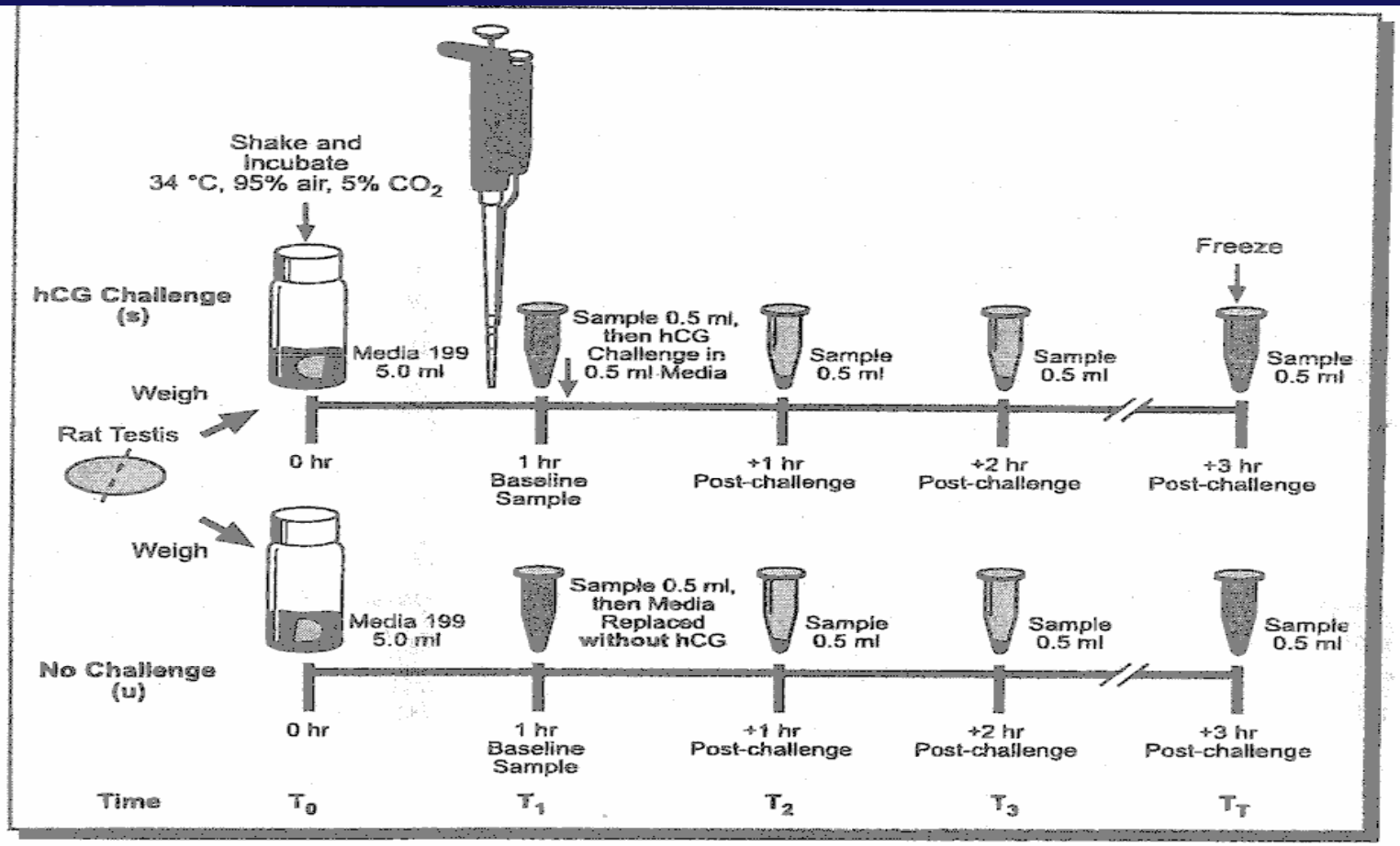

Figure 2. Technical Flow Illustration of the Sliced Testis Steroidogenesis Assay

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Phase II Optimization

Currently Being Analyzed

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